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Im Auftrag

For the President of the European Patent Office

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Use of proline-specific endproteases to hydrolysate peptides and proteins

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USE OF PROLINE SPECIFIC ENDOPROTEASES TO HYDROLYSE PEPTIDES AND PROTEINS

Field of the invention

The present invention relates to the proteolytic hydrolysis of a peptide or protein.

Background of the invention

Proteins in bovine milk are associated with growth and health and form an important ingredient in the human diet. Casein constitutes approximately 80% of the total protein in bovine milk and is an important source of amino acids, calcium and phosphate. Casein consists of roughly 50% of alpha-caseins, 35% of beta-caseins, 13% of kappa-caseins and 3% of gamma-caseins. In human milk the alpha-casein fraction is generally absent.

It is known that upon metabolisation of casein a number of new bioactive peptides are formed. From the alpha and beta-casein fractions opioid peptides called alpha-casomorphins and beta-casomorphins, respectively, have been identified and isolated. The pharmacological effects of especially the beta-casomorphins have been extensively studied. The beta-casomorphin with the sequence Tyr-Pro-Phe-Pro-Gly-Pro-Ile is the principal opioid peptide in bovine milk and is called BCM-7 (beta-casomorphin (1-7); Chang *et al.*(1985) Journal of Biological Chemistry, 260, 9706-9712). Apart from this BCM-7 fragment at amino acid positions 60-66 of the beta-casein molecule, smaller fragments of BCM-7 like Tyr-Pro-Phe-Pro (beta-casomorphin (1-4)) and Tyr-Pro-Phe-Pro-Gly (beta-casomorphin (1-5)) at amino acid positions 60-63 and 60- 64 respectively as well as all larger BCM-7 related peptides up to a chain length of 11 amino acids (at amino acid positions 60-70) display at least some degree of opioid activity. The N-terminal tripeptide of BCM-7, i.e. the sequence Tyr-ProPhe at position 60-62, has no opioid activity. A genetic beta-casein variant caled A1 (having a histidine rather than the proline residue of A2 beta-casein at amino acid position 67) is claimed to lead to the formation of increased levels of the BCM-7 molecule.

The basic reason for the generation of the various beta-casomorphins is that their amino acid sequence is relatively rich in proline residues. Because peptide bonds involving proline residues resist proteolytic breakdown, the beta-casomorphin sequences tend to survive exposure to the gastrointestinal proteases in the stomach and the intestinal lumen. For the same reason one may assume that these beta-casomorphin sequences tend to survive incubations with other proteases, for example those proteases commonly used in the industrial production of protein hydrolysates. This assumption implies that the commonly available protein hydrolysates or products containing these protein hydrolysates all contain the BCM-7 or closely related peptides. As the BCM-7 peptide fragment and its related molecules have been linked with certain diseases, the presence of such molecules in protein hydrolysates, quite often used in the diet of vulnerable groups like infants, elderly and patients, is an undesirable situation.

Results of opiate receptor binding assays of human and bovine beta-casomorphins indicate that the fragments with opioid activity bind with opiate receptors in the rat brain membrane. It has been shown that the beta-caseins are more selective towards mu-ligands with little affinity for delta- and kappa-receptor subtypes. According to these and other studies beta-casomorphins are claimed to have various gastrointestinal, analgesic, respiratory, cardiovascular, endocrine and immunomodulatory effects. Although in normal individuals the peptidases in the intestinal wall and in the blood can cope with the beta-casomorphins, this seems not to be always the case for patients with schizophrenia, autism or other mood disorders. Recent scientific literature provides compelling evidence that an incomplete degradation of these protease resistant peptides may contribute to the development and the severity of such diseases. Apart from the caseine derived BCM-7 fragment, also gluten derived protease resistant peptides have been mentioned in this connection.

Already in 1979 Panksepp (Trends in Neuroscience 1979;2:174-177) proposed the opioid excess theory in which he suggested that a disturbed opioid metabolism is part of the pathogenesis in autism. Nowadays several lines of evidence link the inability to properly digest casein or gluten derived proline rich amino acid sequences with diseases like autism, schizophrenia, celiac sprue and other mood disorders.

Gluten is the insoluble protein fraction of cereals like wheat, rye, oats, barley, maize and rice that remains after washing to remove starch and water-soluble components. Gluten can be subdivided into 4 major solubility fractions i.e. albumin, globulin, prolamin and glutelin. Among these especially the prolamin and the glutelin

fractions of wheat, corn, barley and oats are characterized by relatively high contents of the amino acids proline and glutamine. Recent evidence has implicated the proline rich gluten sequences as a factor in the development of celiac disease. Celiac disease, also known as celiac sprue, is an autoimmune disease of the small intestine caused by the ingestion of gluten proteins. It commonly appears in early childhood with severe symptoms like chronic diarrhea and abdominal distension; later in life symptoms include fatigue, weight loss due to malabsorption and neurological symptoms. Among the proline rich fractions of the various cereals, alpha-gliadin from wheat, hordein from barley, secalin from rye and avenin from oats seem to be most toxic (Schuppan,D.; Gastroenterology 2000;119:234-242). Recently Shan *et al* (Science; vol 297, 27 September 2002: 2275-2279) identified a gliadin-derived, proline rich, 33 amino acids long peptide thought to be the source of a set of major celiac patientspecific T cell epitopes. Whereas an enzyme extract prepared from small intestine brush-border cells was unable to hydrolyse this 33-mer, suppletion with a bacterial prolyl oligopeptidase from *Flavobacterium meningosepticum* led to a rapid digestion with a concomitant strongly decreased stimulation of a relevant Tcell clone. The article indicates the potential of the enzyme in detoxifying gluten by enzyme therapy.

Prolyl oligopeptidases (EC 3.4.21.26) have the unique possibility of preferentially cleaving peptides at the carboxyl side of proline residues. In the prolyl oligopeptidases isolated from mammalian sources as well as in the prolyl oligopeptidase isolated from *Flavobacterium meningosepticum* a unique peptidase domain has been identified that excludes large structured peptides from the enzyme's active site. In fact these enzymes are unable to degrade peptides containing more than about 30 amino acid residues so that these enzymes are now referred to as "prolyl *oligopeptidases*" (Fulop *et al* : Cell, Vol. 94, 161-170, July 24,1998). All known prolyl oligopeptidases are cytosolic enzymes that exhibit pH optima near neutrality and are characterized by the fact that they cannot efficiently degrade peptides containing more than approximately 30 amino acid residues.

Another enzyme that can have a benefit in the inactivation of pathogenic peptides, is the enzyme dipeptidyl peptidase IV (US2002/0041871A). Dipeptidylpeptidase IV, also called Xaa-Pro-dipeptidyl-aminopeptidase (EC 3.4.14.5) catalyzes the release of an N-terminal dipeptide, Xaa-Xbb+Xcc, preferentially when Xbb is proline and provided Xcc is not proline. Dipeptidyl-peptidase IV has been isolated from a large number of mammalian sources, for example the intestinal brush border membranes form a rich source of the enzyme. Furthermore the enzyme has been

isolated from microbial sources such as the food grade microorganisms Saccharomyces, Lactococcus and Aspergillus. All known dipeptidyl-peptidases IV are cytosolic, non-secreted enzymes with near neutral pH optima.

Because of the possible implications of these enzymes in the treatment of celiac disease or schizophrenia, autism or other mood disorders, these data have resulted in a number of patent applications that deal with various aspects of this matter. For example US 6,447,772 and WO 01/24816 describe compositions containing dipeptidyl peptidase IV, WO 02/45523 describes low allergenic protein hydrolysates prepared with proline specific endoproteases and WO 03/028745 describes compositions comprising bacterial strains that can lower the concentration of intestinal pathogenic peptides. WO 96/36239 describes the advantages of products derived from cattle substantially free of the beta-casein A1 allele.

Summary of the invention

The present invention relates to a process for the proteolytic hydrolysis of a peptide or protein, said peptide comprising 4 to 40, preferably 5 to 35, amino acid residues and whereby the amino acid residues of the peptide or protein consist for at least 30%, preferably for at least 40%, of proline residues whereby said peptide is hydrolysed by a proline specific endoprotease (or peptidase) at a pH of 6.5 or lower to hydrolyse of said peptide or protein. Preferably 80% of said peptide is hydrolysed.

Also the present invention relates to a process for the proteolytic hydrolysis of said peptides or protein present in milk proteins obtained from cattle carrying the beta-casein A1 or the beta-casein A2 allele.

Furthermore the use of a proline specific endoprotease having a pH optimum below 6.5 to hydrolyse a peptide comprising 4 to 40, preferably 5 to 35, amino acid residues, or a protein and whereby the amino acids of said peptide or protein consist for at least 30%, preferably for at least 40% of proline residues, is disclosed.

Moreover the present invention provides proline specific endoprotease for use as a medicament, and the use of proline specific endoprotease for the manufacture of a medicament for treatment or preventing autism, schizophrenia, celiac sprue, to defer or to minimise the phenomena of a disturbed opioid metabolism, heart disease and diabetes.

Detailed description of the invention

Several publications point towards the possibilities of using enzymes like dipeptidyl peptidase IV and prolyl oligopeptidase in preventing a disturbed opioid metabolism. These enzymes can become active only after a significant pre-degradation of the gluten or casein molecules by other, for example gastrointestinal endoproteases. Because the required extensive degradation, only limited time is available before consumed food enters the small intestine. Therefore there is a need for an enzyme that would be able to cleave peptides at the carboxyl side of proline residues and also be able to cleave intact proteins or peptides of more than 30 amino acid residues at the carboxyl side of proline residues. However up to now such an enzyme is not known.

WO 02/45524 describes a proline specific endoprotease obtainable from *Aspergillus niger*. Surpsrisingly we have found now that this *Aspergillus* enzyme can hydrolyse intact proteins, large peptides as well as smaller peptide molecules. By the proline specific endo protease accoding to the invention or used according to the invention is meant the polypeptide as mentioned in claims 1-5, 11 and 13 of WO 02/45524. Therefore this proline specific endo protease is a polypeptide which has proline specific endoproteolytic activity, selected from the group consisting of:

(a) a polypeptide which has an amino acid sequence which has at least 40% amino acid sequence identity with amino acids 1 to 526 of SEQ ID NO:2 or a fragment thereof;

(b) a polypeptide which is encoded by a polynucleotide which hybridizes under low stringency conditions with (i) the nucleic acid sequence of SEQ ID NO:1 or a fragment thereof which is at least 80% or 90% identical over 60, preferably over 100 nucleotides, more preferably at least 90% identical over 200 nucleotides, or (ii) a nucleic acid sequence complementary to the nucleic acid sequence of SEQ ID NO:1. The SEQ ID NO:1 and SEQ ID NO:2 as shown in WO 02/45524. Preferably the polypeptide is in isolated form.

The prefered polypeptide has an amino acid sequence which has at least 50%, preferably at least 60%, preferably at least 65%, preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least about 97% identity with amino acids 1 to 526 of SEQ ID NO: 2 or comprising the amino acid sequence of SEQ ID NO:2.

Preferably the polypeptide is encoded by a polynucleotide that hybridizes under low stringency conditions, more preferably medium stringency conditions, and most preferably high stringency conditions, with (i) the nucleic acid sequence of SEQ ID NO:1 or a fragment thereof, or (ii) a nucleic acid sequence complementary to the nucleic acid sequence of SEQ ID NO: 1.

The term "capable of hybridizing" means that the target polynucleotide of the invention can hybridize to the nucleic acid used as a probe (for example, the nucleotide sequence set forth in SEQ. ID NO: 1, or a fragment thereof, or the complement of SEQ ID NO: 1) at a level significantly above background. The invention also includes the polynucleotides that encode the proline specific endoprotease of the invention, as well as nucleotide sequences which are complementary thereto. The nucleotide sequence may be RNA or DNA, including genomic DNA, synthetic DNA or cDNA. Preferably, the nucleotide sequence is DNA and most preferably, a genomic DNA sequence. Typically, a polynucleotide of the invention comprises a contiguous sequence of nucleotides which is capable of hybridizing under selective conditions to the coding sequence or the complement of the coding sequence of SEQ ID NO: 1. Such nucleotides can be synthesized according to methods well known in the art.

A polynucleotide of the invention can hybridize to the coding sequence or the complement of the coding sequence of SEQ ID NO:1 at a level significantly above background. Background hybridization may occur, for example, because of other cDNAs present in a cDNA library. The signal level generated by the interaction between a polynucleotide of the invention and the coding sequence or complement of the coding sequence of SEQ ID NO: 1 is typically at least 10 fold, preferably at least 20 fold, more preferably at least 50 fold, and even more preferably at least 100 fold, as intense as interactions between other polynucleotides and the coding sequence of SEQ ID NO: 1. The intensity of interaction may be measured, for example, by radiolabelling the probe, for example with ^{32}P. Selective hybridization may typically be achieved using conditions of low stringency (0.3M sodium chloride and 0.03M sodium citrate at about 40°C), medium stringency (for example, 0.3M sodium chloride and 0.03M sodium citrate at about 50°C) or high stringency (for example, 0.3M sodium chloride and 0.03M sodium citrate at about 60°C).

The UWGCG Package provides the BESTFIT program which may be used to calculate identity (for example used on its default settings).

The PILEUP and BLAST N algorithms can also be used to calculate sequence identity or to line up sequences (such as identifying equivalent or corresponding sequences, for example on their default settings).

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis ofthe similarity between two sequences. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

The polypeptide is preferably obtained from a fungus, preferably an *Aspergillus*, more preferably from *Aspergillus niger*. So, the *Aspergillus* derived enzyme is found to be a true endoprotease. Proline specific oligopeptidases in general including the enzyme as can be obtained from *Flavobacterium meningosepticum* were found to efficiently hydrolyse smaller peptides only, i.e.upto a length of approximately 30 amino acid residues.

The present invention provides economical, food grade compositions to defer or to minimise the phenomena of a disturbed opioid metabolism. The compositions include oral enzyme formulations suited for alimentary, pharmaceutical and veterinary use as well as enzyme formulations suited for the production of protein hydrolysates.

The present invention discloses methods to hydrolyse proline rich peptides or proteins which are brought in relation with diseases like autism, schizophrenia, celiac sprue and other mood, mental or neurological disorders, heart disease and diabetes. An embodiment of the present invention is related to the breakdown of these proline rich peptides or proteins before consumption. Another embodiment of the invention relates to the suppletion of a suitable enzyme for the breakdown in the body (human or animal) of these proline rich peptides or proteins. In the latter way persons suffering from these diseases and/or having decreased level of body proline specific proteases required for the breakdown of these peptides or proteins, are capable to degrade the relevant proline rich peptides or proteins.

Preferably at least 80% of the proline rich peptides which are formed upon an incubation of peptides or protein with subtilisin (EC 3.4.21.62) preferably a *Bacillus lichenisformis* subtilisin (or subtilisin Carlsberg) are hydrolysed by the proline specific endoprotease according to the invention. The formation of such subtilisin resistant peptides is illustrated in Example 4 of this application. By proline rich is to be understood that the amino acid residues of the protein or peptide comprise for at least 30%, preferably for at least 40% of proline residues. Such subtilisin resistant proline rich peptides are often related with the diseases mentioned above. Examples of these peptides are BCM-7, BCM-7 related peptides i.e. peptides comprising the amino acid sequence YPFP as present at position 60 to 63 of the beta-casein molecule. Furthermore gliadin derived peptides comprising one or more amino acid sequences of QPQLP (for example the PYPQPQLPY epitope) as well as other subtilisin resistant molecules that can be obtained from gliadin, hordein, secalin or avenin are examples hereof. More preferably at least 90%, still more preferably at least 95% and most preferably at least 99% of proline rich peptides which would be formed by hydrolysis by subtilisin are broken down or not formed by using the proline specific endoprotease according to the invention.

One way of supplying the required enzymes would be in the form of a digestive aid, e.g. as stabilized enzyme formulations that are coingested with the food to help the gastro-intestinal digestion of dietary proline rich peptides or proteins. Another way would be to prevent the ingestion of the problematic proline rich sequences, e.g. by using

protein food "pre-digested" with *Aspergillus* enzyme. Such protein food could be supplied in the form of a hydrolysate, e.g. a gluten or a milk protein hydrolysate and includes hydrolysates that have been extensively digested by endo- as well as exoproteases to release large quantities of free amino acids A typical example of the latter application would be the generation of gluten hydrolysates rich in glutamate for a.o. savoury applications.The hydrolysate could be consumed as such or could serve as a food ingredient. In such an application the proline specific enzyme would be used as a so-called processing aid.

The strains of the genus *Aspergillus* have a food grade status and enzymes derived from these micro-organisms are known to be from an unsuspect food grade source. According to another preferred embodiment, the enzyme is secreted by its producing cell rather than a non-secreted, socalled cytosolic enzyme. In this way enzymes can be recovered from the cell broth in an essentially pure state without expensive purification steps. Preferably the enzyme has a high affinity towards its substrate under the prevailing pH and temperature conditions.

In the use as digestive aid, the enzyme should be sufficiently active at a temperature of 37 degrees C and should preferably have a low pH optimum to survive the acid conditions in the stomach. According to published data the acidty of ingested food decreases from an initial pH 5 value to pH 3.5 thirty minutes after ingestion followed by a further decrease to pH 2 sixty minutes after ingestion (thesis Mans Minkus; University of Utrecht, The Netherlands; ISBN:90-393-1666-X).

If used as industrial processing aid in the production of protein hydrolysates, the enzyme should be sufficiently active under conditions that allow microbially safe incubations under non-sterile industrial conditions. Adequate enzyme activity at a processing temperature of at least 50 degrees C and a pH value well below pH 5.5 meets these requirements.

The basidiomycete *Agaricus bisporus* (Sattar *et al*; J. Biochem. 107, 256-261 (1990)) and the non-related ascomycete *Aspergillus niger* (WO 02/45524) have both been shown to produce an extracellular prolyl endopeptidase. However, the enzyme obtained from the basidiomycete will not survive pH values below 5 and is therefore less atractive. Preferably a prolyl oligopeptidase from *A. niger* is used which has an acid pH optimum.

The present invention provides enzyme preparations which combine low costs, legislative acceptance with a proven efficacy under acid pH conditions towards proline

rich peptide sequences. Preferably the same enzyme can be used to degrade not only the A1 as well as the A2-type beta-casomorphins but also various gluten epitopes.

This *Aspergillus* proline specific endoprotease is found to be very active in breaking down proline rich proteins. Advantageously this enzyme is secreted into the fermentation broth, has an acid pH optimum and can be produced food grade and in an economic way. The relevant beta-casomorphin peptides contain up to four proline residues in the molecule and, moreover, the A1 and the A2 beta-casomorphins have different amino acid sequences. Quite surprisingly we have found that the *Aspergillus* enzyme is capable of hydrolysing beta-casomorphins at the C-terminal side of the proline at position 61 and thus effectively inactivates all BCM-7 and BCM-7 related peptide, both for A1 or A2 beta-casein. This is quite remarkable because we have found that a widely used and highly aggressive endoprotease with a broad substrate specificity such as subtilisin (EC 3.4.21.64) commercially available as for example Alcalase, is not able to degrade BCM-7. In fact the *Aspergillus* derived proline specific endoprotease can hydrolyse beta casomophine but at only one of the four proline residues. Nevertheless, because of the specificity of this particular *Aspergillus* enzyme, BCM-7 as well as all BCM-7 related molecules are effectively destroyed by incubation with this enzyme. Moreover, BCM-7 molecules derived from A1 as well as from A2 beta-caseins are inactivated by hydrolysis of this the unique cleavage site of these proteins by the *Aspergillus* proline specific endoprotease.

The advantage of a true proline specific endoprotease is that the proline specific endoprotease can start hydrolysing proline rich sequences immediately upon contacting the enzyme with the protein. Prolyl oligopeptidases can become active only after a significant pre-degradation of the gluten or casein molecules by other, for example gastrointestinal endoproteases. In view of the required extensive degradation and the limited time available before the food enters the small intestine, a true proline specific endoprotease has significant application advantages over the known oligopeptidases. Another advantage of a true proline-specifc endoprotease is that it can be used to reduce the level of pathogenic peptides in gluten without a total destruction of the gluten structure. For example extruding a wheat gluten paste together with the *Aspergillus* derived enzyme will yield a product with some residual textural properties but with a strongly reduced level of pathogenic peptides. To achieve the same reduction of pathogenic peptides with a prolyl oligopeptidase an almost total pre-hydrolysis with other proteases would be required to achieve the desired

peptide lengths below 30 amino acid residues. Needless to say that this will result in a complete loss of all relevant physico-chemical properties of the gluten.

Collectively these considerations indicate novel and considerable advantages for the *A. niger* derived enzyme over the enzymes mentioned in the prior art. Compositions containing the enzymes according to the invention are advantageously used to reduce or delay the phenomena of a disturbed opioid metabolism. Such compositions can be applied as a digestive aid to achieve a gastrointestinal *in situ* reduction of the pathogenic peptides. Alternatively such compositions can be applied as a processing aid to produce protein hydrolysates without such pathogenic peptides.

Example 1 of the present application shows the acidic pH optimum and an ideal temperature optimum of the *Aspergillus* derived proline specific endoprotease. In Examples 2 and 3 we show that the proline specific endoprotease producible by *Aspergillus niger* is a true proline specific endoprotease that can cleave large, intact proteins with the same efficiency as smaller peptides. In fact our data indicate that the *Aspergillus* enzyme is a new member of the S28 family rather than the S9 family to which the known oligopeptidases belong (N.D. Rawlings and A.J. Barrett, Methods in Enzymology, Vol. 244, pp 1961, 1994; N.D. Rawlings and A.J. Barrett, Biochimica & Biophysica Acta 1298(1996) 1-3). We have found that, in contrast with the known prolyl oligopeptidases, the *Aspergillus* derived prolyl endoprotease survives the acidic conditions in the stomach and shows high efficiencies towards the hydrolysis of large proline rich protein fragments. Such high efficiencies are illustrated in Examples 4, 5 and 6. In Example 4 we demonstrate that during the production of milk protein hydrolysates with Alcalase, an aggressive broad spectrum protease frequently used in the production of protein hydrolysates, several peptides incorporating BCM-7 sequences survive the hydrolysis process. However, these peptides rapidy disappear upon an incubation under acid conditions with the *Aspergillus* derived prolyl endoprotease. The data provided in Example 5 reveal the surprising fact that the Aspergillus enzyme cleaves only one of the four proline residues available. This indicates that the incubation of a proline rich substrate with any proline- specific protease does not automatically imply the cleavage of all peptide bonds involving a proline residue, not even under conditions of a dramatically increased enzyme/ substrate ratio. In Example 6 we demonstrate the efficacy of the *Aspergillus* derived prolyl endoprotease towards the gliadin derived 33-mer claimed to be a major epitope in celiac patients. Although again the broad spectrum Alcalase cannot cleave this molecule, neither under alkaline nor under acid conditions, the Aspergillus derived enzyme frequently cleaves the molecule under acid

conditions generating 99.5% peptides with a maximum length of 6 amino acid residues. So, despite its high efficacy towards proline rich peptides under acid conditions, even the *Aspergillus* derived enzyme leaves at least 0.5% of a heptamer with the amino acid sequence YPQPQLP. As the sequence PYPQPQLPY is a known celiac patient-specific T cell epitope, this finding illustrates that for subopfimal proline specific enzymes such as the known proline specific oligopeptidases including the enzyme derived from *Flavobacterium meningosepticum* a realistic *in vivo* application to prevent the formation of toxic peptides from gluten molecules will proof to be impossible.

Gluten is a non-water soluble compound with a complex three dimensional structure. These properties in combination with its proline rich amino acid composition make the gluten molecules resistant to gastric and intestinal proteolysis. As none of the natural proteolytic activities secreted into the intestinal lumen is capable of cleaving peptide bonds involving proline, the use of synergistic exogeneous proline specific enzymes makes sense. However, persons suffering from celiac disease can be extremely sensitive towards the many epitopes that are present in gluten. According to the present invention the effect of the natural digestive proteases can be improved with the *Aspergillus* derived prolyl endoprotease, and even further enhancement of the hydrolytic capacity of this proteolytic mixture is disclosed herein.

It is well known that peptide bonds involving negatively charged residues such as Glu (E) and Asp (D) form poor substrates for proteases. Also the natural gastrointestinal proteolytic enzymes cannot cope with these residues as evidenced by the isolation of the gastric and pancreatic protease resistant peptide WQIPEQSR from gliadin (cf. Shan *et al*). The latter publication also makes clear that the ubiquitously present glutamine residues (Q) in gluten can be deamidated to glutamate residues (E) by tissue transglutaminase. Unfortunately this regiospecific deamidation of gliadin peptides further increases their immunogenic potential. Against this background we have been able to create an effective enzyme combination existing of an *Aspergillus* derived proline specific endoprotease with an auxillary endoprotease to prevent the formation of proline rich pathogenic peptides. According to the present invention glutamate-specific endoproteases (EC3.4.21.19) can be used, for example those glutamate-specific endoproteases that are over secreted by a number of food-grade microorganisms such as *Bacillus* and *Streptomyces*. These enzymes can be produced in an economic and food-grade way. Enzymes which have a safe passage through the stomach, which respect to their enzymatic activity are preferred. In general those enzymes will have an acidic or neutral pH optimum. In combination with the

Aspergillus derived prolyl endoprotease, this category of glutamate-specific endoproteases is considered useful in the production of protein hydrolysates with low levels of pathogenic peptides.

Quite surprisingly our present research demonstrates that apart from the glutamate specific endoproteases other endoproteases exist that have a synergistic effect on incubations with the proline specific endoprotease from *Aspergillus.* We conclude that endoproteases (EC 3.4.21-99) capable of cleaving between the amino acid residues Q (glutamine) and L(leucine) are advantageously combined with the proline specific endopeptidase from *Aspergillus.*

One application of the enzymes according to the invention is their use as a digestive aid. In this application the compositions of the present invention are preferably administered orally, but may also be administered via other direct routes. The compositions are typically administered to human beings but may also be administered to animals, preferably mammals, to relief the symptoms typically for a disturbed casein or gluten metabolism. In their application as digestive aid the enzymes according to the invention may be formulated as a dry powder in, for example, a pill, a tablet, a granule, a sachet or a capsule. Alternatively the enzymes according to the invention may be formulated as a liquid in, for example, a syrup or a capsule. The compositions used in the various formulations and containing the enzymes according to the invention may also incorporate at least one compound of the group consisting of a physiologically acceptable carrier, adjuvant, excipient, stabiliser, buffer and diluant which terms are used in their ordinary sense to indicate substances that assist in the packaging, delivery, absorption, stabilisation, or, in the case of an adjuvant, enhancing the physiological effect of the enzymes. The relevant background on the various compounds that can be used in combination with the enzymes according to the invention in a powdered form can be found in "Pharmaceutical Dosage Forms", second edition, Volumes 1,2 and 3, ISBN 0-8247-8044-2 Marcel Dekker, Inc. Although the enzymes according to the invention formulated as a dry powder can be stored for rather long periods, contact with moisture or humid air should be avoided by choosing suitable packaging such as for example an aluminium blister. If formulated in a liquid form, the compounds used for stabilising the enzyme activity and microbial preservation play an important role. The stabilisation of enzyme activity may require lowered water activities as can be obtained by the use of polyols such as glycerol or various sugars. Moreover, divalent cations such as Ca2+ or Mg 2+ are known for their stabilising effects as well as reducing agents such as sulphur containing amino acids and phenolic compounds such as

BHT or propyl gallate. Food grade microbial preservation may be achieved using well known combinations of low pH conditions or low water activities with sorbate or benzoate or parabenes. Furthermore food grade thickeners such as a hydrocolloid may be required. A relatively new oral application form is the use of gelatin capsule containing a liquid. In this application the liquid is typically an oil or a poly ethylene glycol or a lecithin in which the dried enzymes according to the invention can be suspended.

Description of the Figures

Figure 1: pH optimum of the *A. niger* derived proline specific endoprotease using the synthetic peptide Z-Gly-Pro-pNA as the substrate.

Figure 2: SDS-PAGE of intact ovalbumine and a synthetic 27-mer peptide after incubation with purified *A. niger* derived proline specific endoprotease

Materials and Methods

Materials.

Alcalase® AF 2.4 L was obtained from Novozymes A/S (Bagsvaerd, Denmark).

Synthetic peptides were obtained from Pepscan Systems B.V.(Lelystad, The Netherlands).

Proline- specific endoprotease from *A. niger*.

Overproduction and chromatographic purification of the proline specific endoprotease from Aspergillus niger was accomplished as described in WO 02/45524. The activity of the enzyme was tested on the synthetic peptide Z-Gly-Pro-pNA at 37 degrees C in a citrate/disodium phosphate buffer pH 5. The reaction product was monitored spectrophotometrically at 405 nM. A unit is defined as the quantity of enzyme that provokes the release of 1 µmol of p-nitroanilide per minute under these conditions.

<u>LC/MS analysis</u>.

HPLC using an ion trap mass spectrometer (Thermoquest®, Breda, the Netherlands) coupled to a P4000 pump (Thermoquest®, Breda, the Netherlands) was used in characterising the enzymatic protein hydrolysates produced by the inventive enzyme mixture. The peptides were separated using a PEPMAP C18 300A (MIC-15-03-C18-PM, LC Packings, Amsterdam, The Netherlands) column in combination with a gradient of 0.1% formic acid and 1 mM nonafluoropentaoic acid (NFPA) in Milli Q water (Millipore, Bedford, MA, USA; Solution A) and 0.1% formic acid in acetonitrile (Solution B) for elution. The gradient started at 95% of Solution A and increased to 40% of solution B in

140 minutes and was kept at the latter ratio for another5 minutes. The injection volume used was 50 microliters, the flow rate was 50 microliter per minute and the column temperature was maintained at 30°C. The protein concentration of the injected sample was approximately 50 micrograms/milliliter.

Detailed information on the individual peptides was obtained by using the "scan dependent" MS/MS algorithm, which is a characteristic algorithm for an ion trap mass spectrometer.

Full scan analysis was followed by zoom scan analysis for the determination of the charge state of the most intense ion in the full scan mass range. Subsequent MS/MS analysis of the latter ion resulted in partial peptide sequence information, which could be used for data base searching using the SEQUEST application from Xcalibur Bioworks (Thermoquest®, Breda, The Netherlands). Data banks used were extracted from the OWL.fasta databank, available at the NCBI (National Centre for Biotechnology informatics), containing the proteins of interest for the application used. In those experiments in which well characterized protein substrates such as whey proteins or caseins were measured, the precision of the analysis technique was increased by omitting those MS/MS spectra with a sequence fit of less than 50%.

Angiotensin (M=1295.6) was used to tune for optimal sensitivity in MS mode and for optimal fragmentation in MS/MS mode, performing constant infusion of 60 mg/ml, resulting in mainly doubly and triply charged species in MS mode, and an optimal collision energy of about 35 % in MS/MS mode.

Example 1

The pH and temperature optima of the proline specific endoprotease as obtained from *A. niger*.

The *A. niger* derived proline specific endoprotease was overexpressed in an *A. niger* host, isolated and chromatographically purified using the materials and methods described in WO 02/45524. To establish the pH optimum of the thus obtained enzyme, buffers with different pH values were prepared. Buffers of pH 4.0 –4.5 – 4.8 – 5.0 – 5.5 and 6.0 were made using 0.05 mol/l Na-acetate and 0.02 M CaCl2; buffers of pH 7.0 and 8.0 were made using 0.05 M Tris/HCl buffers containing 0.02 M CaCl2. The pH values were adjusted using acetic acid and HCl respectively. The chromogenic synthetic peptide Z-Gly-Pro-pNA was used as the substrate. The buffer solution, the substrate

solution and the prolyl endoprotease pre-dilution (in an activity of 0.1 U/mL), were heated to exactly 37.0°C in a waterbath. After mixing the reaction was followed spectrophotometrically at 405 nm at 37.0°C for 3.5 min, measuring every 0.5 min. From the results shown in Figure 1 it is clear that the *A. niger* derived proline specific endoprotease has a pH optimum around 4.

Also the temperature optimum of the prolyl endoprotease was established. To that end the purified enzyme preparation was incubated in 0.1 mol/l Na-acetate containing 0.02 mol/l CaCl2 at pH 5.0 for 2 hours at different temperatures using Caseine Resorufine (Roche version 3) as the substrate and enzyme activity was quantified by measuring at 574 nm. According to the results obtained the proline specific endoprotease from *A. niger* has a temperature optimum around 50 degrees C.

The very acidic pH optimum strongly suggests that the *A. niger* derived proline specific endoprotease has ideal properties for industrial application as well as for oral consumption as it will be optimally active under the acidic conditions preferred for industrial application and the conditions prevailing in the stomach and the early part of the small intestine. Also the temperature optimum of the enzyme makes the enzyme ideally suitable for both applications.

Example 2

The enzyme as obtained from *A. niger* represents a new class of proline specific enzymes.

From the entire coding sequence of the *A. niger* derived proline specific endoprotease as provided in WO 02/45524 a protein sequence of 526 amino acids can be determined. The novelty of the enzyme was confirmed by BLAST searches of databases such as SwissProt, PIR and trEMBL. To our surprise, no clear homology could be detected between the *A. niger* enzyme and the known prolyl oligopeptidases. Closer inspection of the amino acid sequence, however, revealed low but significant homology to Pro-X carboxypeptidases (EC3.4.16.2), dipeptidyl aminopeptidases I (EC3.4.14.2), and thymus specific serine protease. All of these enzymes have been assigned to family S28 of serine peptidases. Also the GxSYxG configuration around the active site serine is conserved between these enzymes and the *A. niger* derived endoprotease. Additionally, members of family S28 have an acidic pH optimum, have specificity for cleaving at the carboxy-terminal side of proline residues and are synthesized with a signal sequence

and propeptide just like the *A. niger* derived proline specific endoprotease. Also the size of the *A. niger* enzyme is similar to those the members of family S28. Therefore, the*A. niger* proline specific endoprotease appears to be a member of family S28 of serine proteases rather than the S9 family into which most cytosolic prolyl oligopeptidases including the enzyme obtained from *Flavobacterium meningosepticum* have been grouped. On the basis of these structural and physiological features we have concluded that the *A. niger* enzyme belongs to the S28 rather than the S9 family of serine proteases. An additional feature that discriminates the*A. niger* derived enzyme from the prolyl oligopeptidases belonging to the S9 family is the fact that, unlike the cytosolic prolyl endoproteases belonging to the latter family, the newly identified*A. niger* enzyme is secreted into the growth medium. Sofar only the basidiomycete *Agaricus bisporus* (Sattar *et al*; J. Biochem. 107, 256-261 (1990)) and the non-related ascomycete *Aspergillus niger* (WO 02/45524) have been shown to produce an extracellular prolyl endopeptidase. However, the enzyme obtained from the basidiomycete will not survive pH values below 5 and is therefore far less suitable for industrial application as well as for oral consumption.

This is the first report on the isolation and characterization of a member of family S28 from a lower eukaryote.

Example 3

The *A. niger* derived proline specific endoprotease can hydrolyse large proteins as well as small peptides and is thus a true endoprotease.

Owing to a specific structural feature, prolyl oligopeptidases belonging to the S9 family cannot digest peptides larger than 30 amino acids. This limitation is an obvious disadvantage for an enzyme, which is meant to hydrolyse as quickly and as efficiently as possible all potential proline rich pathogenic peptides. To see if the *A. niger* derived proline specific endoprotease exhibits the same limitations with respect to the size of the substrate molecule, we have incubated the chromatographically purified prolyl endopeptidase from *A. niger* with a small synthetic peptide and with the large ovalbumine molecule and have analysed the hydrolysis products formed by SDS-PAGE. The synthetic peptide used was a 27-mer of the sequence NH2-FRASDNDRVIDPGKVETLTIRRLHIPR-COOH and was a gift of the Pepscan company

(Lelystad,The Netherlands). As shown by its amino acid sequence, this peptide contains 2 proline residues, one in the middle and one at the very end of the peptide.

The intact ovalbumine molecule (Pierce Imject, vials containing 20mg freeze dried material) consists of 385 amino acids with a molecular weight of 42 750 Da. This molecule contains 14 proline residues, one of which is located at the ultimate C-terminal end of the molecule and cannot be cleaved by a proline specific endoprotease.

Ovalbumin and the oligopeptide were separately incubated at 50°C with the purified *A. niger* derived proline specific endoprotease. At several time intervals samples were taken which were the analysed using SDS-PAGE.

A chromatographically purified *A. niger* derived proline specific endoprotease with an activity of 4.5 units/ml was diluted 100-fold with 0.1 M acetate buffer pH 4 containing 20mM CaCl2. The ovalbumine was dissolved in acetate buffer pH 4 to a concentration of 1 mg/ml (22μM). The 27-mer was dissolved in the same buffer to reach a concentration of 0.48 mg/ml (152μM). The molarity of the ovalbumine and the 27-mer solution was chosen in such a way that both solutions contained the same molarity in cleavable proline residues. Ovalbumine contains 13 potential proline cleavage sites, whereas the 27-mer peptide has only two. Of both substrate solutions 0.5 ml was incubated with 10μl (0.45milliU) of the enzyme solution in an Eppendorf thermomixer at 50°C. At several time intervals 10μl samples were withdrawn from the incubation mixture and kept at 20°C until SDS-PAGE. All materials used for SDS-PAGE and staining were purchased from Invitrogen. Samples were prepared using LDS buffer according to manufacturers instructions and separated on 12% Bis-Tris gels using MES-SDS buffer system according to manufacturers instructions. Staining was performed using Simply Blue Safe Stain (Collodial Coomassie G250).

As can be seen in Figure 2 ovalbumine is cleaved by the *Aspergillus* derived enzyme into a discrete band of about 35 to 36kD in the first 4.75 hours of incubation (lane 3). Prolonged incubation periods result in further breakdown to smaller products of various molecular weights (lane 7).

The 27-mer peptide is also broken down, as judged by the more faint bands in lanes 4, 6 and 8 as compared to lane 2. The very small molecular weight shift of the product (compare lanes 9 and 8) is most likely due to cleaving of the arginine residue at the carboxylic end of the peptide. The difference is about 200D (measured using AlphaImager 3.3d software on an AlphaImager 2000 system) and arginine has a MW of

174. This small molecular weight shift is probably the first step in the breakdown of the peptide.

The further decay of the product can only be seen by the decrease in intensity of the band on the SDS gel. The products of further decay are not visible, as in gel staining of components with a MW of about 1000 is not possible with Coomassie Brillant Blue.

From this experiment it can be concluded that, unlike the known prolyl oligopeptidases belonging to the S9 family, the *A. niger* derived proline specific endoprotease has no specific preference for cleaving small sized peptides over much larger proteins. As such the *A. niger* derived enzyme represents a true endoprotease and a preferred enzyme to cleave potential proline rich pathogenic peptides.

Example 4

Beta-casomorphins in hydrolysates formed after incubation with Alcalase and a combination of Alcalase plus proline specific endoprotease from *A. niger*.

In analogy with the formation of protease-resistant beta-casomorphins during gastro-intestinal proteolysis, we wondered whether during the industrial production of milk protein hydrolysates a similar accumulation of BCM-7 related peptide fragments would occur. To that end we incubated A2 beta-casein isolated from bovine milk with the industrially frequently used subtilisin Alcalase and with Alcalase plus the proline specific endoprotease from *A. niger*. Using LC/MS/MS analysis the peptides thus formed were analysed.

Bovine milk contains almost 10 grams of beta-caseine per kg of milk representing 28% of all protein present. To facilitate the analysis of BCM-7 related amino acid sequences, in this experiment we used a concentrated preparation (from Sigma) containing a minimum of 90 % (A2)beta-casein. The latter product was dissolved in water in a concentration of 20 grams per litre after which the pH was adjusted to 8 using NaoH and Alcalase was added in an amount of 800 microlitre of enzyme concentrate per litre of casein solution. Incubation was carried out for 2 hours at 60 °C. Then the pH of the solution was lowered till 4.5 using citric acid. The solution was then split into two parts: one part was heated for 5 minutes at 90 °C to inactivate the Alcalase and to the other part the *A. niger* derived proline specific endoprotease was added to obtain an enzyme concentration of 1 unit per gram of casein present (see Materials &Methods section for unit definition). Incubation with the *A. niger* derived proline specific

endoprotease was continued for 16 hours at 55 °C followed by another heat treatment to inactivate the proline specific endoprotease. Finally two samples with an estimated beta-casein concentration of 20 mg/ml were supplied for LC/MS/MS analysis. The two samples were centrifuged for 10 minutes at 13000 rpm and diluted 20 times in Milli Q prior to LC/MS/MS analysis. LC/MS/MS analysis was carried out as described in the

Materials &Methods.

Apart from the BCM-7 sequence Tyr-Pro-Phe-Pro-Gly-Pro-Ile at amino acid positions 60-66 of the beta-casein molecule, the smaller fragments like Tyr-ProPhe-Pro (beta-casomorphin (1-4)) and Tyr-Pro-Phe-Pro-Gly (beta-casomorphin (1-5)) at amino acid positions 60-63 and 60- 64 respectively as well as all larger peptides up to a chain length of 11 amino acids (at amino acid positions 60-70) display at least some degree of opioid activity. The tripetide Tyr-Pro-Phe at position 60-62 has been reported to have no opioid activity.

For the peptide identification direct LC/MS/MS of the protonated molecules was used. The protonated masses of the possibly relevant peptides are provided in Table 1. All spectra were obtained with a collision amplitude of 35% and a peak width of 3 Da. All experimental data are compared with the theoretical fragmentation pattern based on the so-called B and Y ions. This is the process normally performed using automatic data processing.

Table 1: Peptide masses analyzed in LC/MS/MS mode.

Peptide amino acid sequence	*m/z*
YPFPGPI	790.4
FPGPIPNS	828.4
YPFPGPIP	887.5
VYPFPGPI	889.5
VYPFPGPIP	986.5
LVYPFPGPI	1002.5
YPFPGPIPNS	1088.5
LVYPFPGPIP	1099.6
VYPFPGPIPN	1100.6
VYPFPGPIPNS	1187.6
YPFPGPIPNSL	1201.7
LVYPFPGPIPN	1213.6
VYPFPGPIPNSL	1300.7
LVYPFPGPIPNSL	1413.8

Betacasomorphine amino acid sequences start with tyrosine (Y; residue nr 60 of the beta-casein peptide chain) and are given in bold. The m/z values represent the protonated molecules of other possibly relevant peptides. The detection of a proline residue at position 67 indicates that the substrate used represents A2 beta-casein.

The results obtained upon LC/MS/MS analysis of the beta-casein hydrolysates obtained after incubation with either Alcalase or Alcalase plus proline specific endoprotease are given in Table 2 and can be summarized as follows:

-The exact beta-casomorphine sequences are not present in (A2) beta-casein treated with either Alcalase or the combination of Alcalase plus the proline specific endoprotease from *A. niger*.

-However, two peptides containing the beta-casomorphin sequence are present in the Alcalase-treated sample i.e. LVYPFPGPIPN and VYPFPGPIPN.

-The intensity of these beta-casomorphin containing sequences are drastically reduced upon treatment with the proline specific endoprotease from *A. niger*.

Table 2: LC/MS/MS identification of peptides containing the beta-casomorphin (1-7) amino acid sequence

Peptide amino acid sequence	*m/z*	Intensity after Alcalase treatment	Intensity after Alcalase + prol.spec endoprotease treatment
YPFPGPI	790.4	——	——
FPGPIPNS	828.4	——	——
YPFPGPIP	887.5	——	——
V**YPFPGPI**	889.5	——	——
V**YPFPGPI**P	986.5	——	——
LV**YPFPGPI**	1002.5	——	——
YPFPGPIPNS	1088.5	——	——
LV**YPFPGPI**P	1099.6	——	——
V**YPFPGPI**PN	1100.6	$100\ 10^7$	$0.05\ 10^7$
V**YPFPGPI**PNS	1187.6	——	——
YPFPGPIPNSL	1201.7	——	——
LV**YPFPGPI**PN	1213.6	$3.5\ 10^7$	——
V**YPFPGPI**PNSL	1300.7	——	——
LV**YPFPGPI**PNSL	1413.8	——	——

The results clearly indicate that the combination of Alcalase plus the proline specific endoprotease from *A. niger* destroys all potential beta-casomorphin sequences with a

high efficiency hereby offering a hydrolysate without potentially pathogenic proline rich sequences.

After a more precise search among the peptides formed we were able to demonstrate the presence of peptide VYP in the hydrolysate formed by the combination of the two enzymes. As this peptide could not be traced in the hydrolysate formed by using just Alcalase, this finding suggests cleavage C-terminal of the proline residue in position 61 of the beta-casein molecule by the *Aspergillus* derived enzyme.

Example 5

Peptides formed upon the incubation of the Alcalase formed peptide VYPFPGPIPN with the proline specific endoprotease from *A. niger*.

As shown in Example 4, the hydrolysis of A2 beta-casein with a combination of Alcalase and the proline specific endoprotease from *A. niger*, effectively removes all potential beta-casomorphin sequences. However, the complexity of the peptides generated did not allow us to establish at which position the *Aspergillus* derived enzyme cleaves the Alcalase formed peptide VYPFPGPIPN. To that end a peptide with this specific sequence was synthesized and incubated with two concentrations of the proline specific endoprotease. Subsequent LC/MS/MS analysis of the peptides formed revealed the exact cleavage site of the enzyme.

The lyophilised 10-mer (Pepscan Systems; Lelystad, The Netherlands) was dissolved in a citrate-phosphate buffer pH 4.5 in a concntration of 2mg/ml. To the solution proline specific endoprotease was added in concentrations of 1 and 10 units per gram of peptide. Incubation took place for 4 hours at 55 degrees C after which a heat treatment of 5 minutes at 90 degrees C was used to inactivate the enzyme. The two samples were then centrifuged for 10 minutes at 13000 rpm and diluted 20 times in Milli Q water prior to LC/MS and LC/MS/MS analysis. The samples were first analyzed in LC/MS mode to observe the decrease in intensity of the 10-mer using different amounts of enzyme and to observe which peptide masses appeared in the LC/MS ion chromatogram after enzymatic cleavage.

Then direct LC/MS/MS of the protonated molecules of the peptides found in the LC/MS runs was performed. All spectra were obtained with a collision amplitude of 35 % and a peak width of 3 Da. All experimental data are compared with the theoretical

fragmentation pattern based on the so-called B and Y ions. This is the process normally performed using automatic data processing for identification of peptides and proteins.

Treatment of the 10-mer VYPFPGPIPN (M= 1099.5) with 1 unit/g of protein already resulted in total breakdown of the 10-mer into several peptides. The intensity of the protonated molecule, at *m/z* 1100.5, drops 3 orders of magnitude. Treatment of the 10-mer with 10 units/g did not result in further decrease of the intensity of the protonated molecule and also no other peptide masses were found. Upon enzymatic treatment with 1unit/g 4 peptides were formed, with VYP (M=377.2), characterized by *m/z*378.2 as the most abundant (almost 98 %; see Table 3). All four peptides were analyzed in LC/MS/MS mode and found to be correct, based on the criteria described above.

Table 3: Protonated peptide masses analyzed in LC/MS and LC/MS/MS mode of the 10-mer VYPFPGPIPN M=1099.5. The second column presents the m/z values of the protonated molecules, the third column the intensity of the protonated molecules observed in LC/MS mode, the fourth column the percentage based on peak area of the protonated molecule and the fifthcolumn the position of the peptides found in the total amino acid sequence of the 10-mer.

Peptide amino acid sequence	*m/z*	*Intensity in LC/MS mode*	*Percentage (%)*	*Position in total aa sequence*
VYP	378.2	$1\ 10^8$	97.7	1-3
VYPF	525.3	$3\ 10^5$	0.3	1-4
VYPFP	622.4	$2\ 10^6$	2.0	1-5
VYPFPGP	776.4	$3\ 10^4$	0.03	1-7

It can be concluded that the proline specific endoprotease from *A.niger* cleaves almost exclusively at the C-terminal side of the proline at position 61 (position 3 for this particular decapeptide. The cleavage performance is not influenced by increasing enzyme/substrate ratios.

As all known beta-casomorhin molecules with opioid activity share the N-terminal sequence YPF, it is evident that the efficient cleavage of this sequence between P and F (i.e. carboxyterminal of the proline residue at position 61) by the proline specific

endoprotease will effectively inactivate all BCM-7 and BCM-7 related peptides, be it from the A1 or from the A2 genetic variant of beta-casein.

The crucial role of the proline residue in position 61 in the interaction with the mu-receptor was also confirmed in a recent internet publication ("Sintesi e affinita ai recettori oppiodi di analoghi delle beta-casomorphine contenenti beta-omo amminoacidi" by the Dipartimento di Scienza degli Alimenti Universita di Napoli"Frederico II" Facolta di Agraria).

Example 6

Peptides formed upon the incubation of the gliadin derived 33-mer LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF with the proline specific endoprotease from *A. niger.*

Treatment of the gastric and pancreatic juices resistant gliadin derived 33-mer LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (M=3911) as described by Shan *et al* (Science, Vol 297, 27 September 2002) with Alcalase at either pH 8 or pH 5 did not result in any cleavage of the molecule. However, similar to the situation with the beta-casein derived 10-mer, incubation with 1 unit of the proline specific endoprotease from *A. niger* at pH 5 resulted in total breakdown of the molecule into several peptides. The intensity of the triple protonated 33-mer at *m/z* 1304.4, drops 3 orders of magnitude. No further decrease of the intensity of the protonated molecule and also no other peptide masses were observed upon treatment of the 33-mer with 10 enzyme units per gram of protein.

After enzymatic treatment about 6 main peptides and several minor peptides were formed, with a peptide characterized by *m/z* 565.2 as the most abundant. All 6 peptides were analyzed in LC/MS/MS mode and they all were found to contain proline at the C-terminus, confirming the enzyme's specificity. The major peptide formed is characterized by *m/z* 565.2 (sequence ..QLP in table 4). Although the C-terminal sequence "LP" could be unabiguously demonstrated for this peptide, the identified mass can theoretically not be formed by endoproteolytic degradation of the 33-mer so that there remains some uncertainty regarding the exact N-terminal composition of the peptide. Possibly this difficulty is related to the repeating (3x) character of the amino acid sequence QPQLP.

Appearance of QPQLP: LQLQPFP<u>QPQLP</u>YP<u>QPQLP</u>YP<u>QPQLP</u>YPQPQPF

Appearance of QPQLPYP : LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF.

The LC/MS/MS spectrum of the peptide with *m/z* 679 could be be elucidated to be PQPQLP. Despite the fact that the nature of the peptide with *m/z* 565.2 was not fully understood, the data obtained clearly demonstrate the preferential cleavage of the proline specific endoprotease from *Aspergillus* at the C-terminal side of the proline residues at positions 12,19 and 26 (i.e. exclusively between the proline and the tyrosine residue) of this particular 33-mer. This cleavage pattern is not influenced by using higher enzyme/substrate ratios. In table 4 all relevant information is summarized.

Table 4: Peptide masses analyzed in LC/MS and LC/MS/MS mode of the 33-mer. The first column specifies the derived peptide sequences, dots used (also in column 5) indicate that no exact starting position of the peptide could be given due to unexplained mass discrepancies. The second column presents the m/z values of the protonated molecules, the third column the intensity of the peptides observed in LC/MS mode, the fourth column the percentages based on peak area of the protonated molecule and the fifth column the position of the peptides identified in the total amino acid sequence.

Peptide amino acid sequence	*m/z*	*Intensity in LC/MS mode*	*Percentage*	*Positions in total aa sequence*
...YP	523.2	$1.9\ 10^7$	5.2	..14, ..21, ..28
YPQPQLP	842.3	$1.7\ 10^6$	0.5	13-19,20-26
QPQP	468.2	$3.4\ 10^7$	9.3	29-32
PQPQLP	679.2	$3.4\ 10^7$	9.3	14-19, 21-26
..QLP	565.2	$2.4\ 10^8$	65.9	..12, ..19, ..26
...P	599.2	$3.5\ 10^7$	9.6	

Cleavage of the 33-mer, claimed to be a major epitope in celiac patients, cannot be accomplished by gastric or pancreatic juices or by incubation with the aggressive broad spectrum protease Alcalase, neither under alkaline nor under acid conditions. Nevertheless our results indicate an efficient cleavage by the proline specific *A. niger* derived endoprotease under acid conditions. The latter cleavage takes place exclusively between the proline and the tyrosine residues of the molecule and generates 99.5% of peptides with no more than 6 amino acid residues long. So, despite its high efficacy

towards proline rich peptides under acid conditions, even the *Aspergillus* derived enzyme leaves at least 0.5% of a heptamer with the amino acid sequence YPQPQLP. As the sequence PYPQPQLPY is a known celiac patient-specific T cell epitope, this finding emphasizes once more that for suboptimal proline specific enzymes with near neutral pH optima such as the known proline specific oligopeptidasesand the enzyme derived from *Flavobacterium meningosepticum*, a realistic *in vivo* application to prevent the formation of toxic peptides from gluten molecules will proof to be impossible.

CLAIMS

1. A process for the proteolytic hydrolysis of a peptide or a protein, said peptide comprising 4 to 40, preferably 5 to 35, amino acid residues, and whereby the amino acid residues of the peptide or protein consist for at least 30%, preferably for at least 40%, of proline residues whereby said peptide or protein is hydrolysed by a proline specific endo protease at a pH of 6.5 or lower to hydrolyse said peptide or protein.

2. A process according to claim 1 wherein a proline specific endo protease derived from *Aspergillus* or belonging to the S28 family of serine proteases is used.

3. A process according to claim 1 or 2 wherein said peptide is not hydrolysable by subtilisin as described in example 4.

4. Use of a proline specific endo protease having a pH optimum below 6.5 to hydrolyse a peptide comprising 4 to 40, preferably 5 to 35, amino acid residues or a protein and whereby the amino acid residues of the peptide or protein consist for at least 30%, preferably for at least 40% of proline residues.

5. Use of claim 4 wherein said peptide is not hydrolysable by subtilisin.

6. Use of a proline specific endoprotease to breakdown proline rich peptides which are brough in relation with diseases like autism, schizophrenia, celiac sprue, mood disorders, heart disease and diabetes.

7. Proline specific endoprotease for use as a medicament.

8. Proline specific endoprotease of claim 7 which is an *Aspergillus* , preferably an *Aspergillus niger* enzyme.

9. Use of proline specific endoprotease for the manufacture of a medicament for treatment or preventing autism, schizophrenia, mood disorders, celiac sprue or to defer or to minimise the phenomena of a disturbed opioid metabolism, heart disease and

diabetes.

10. Use of proline specific endoprotease to hydrolyse protein or peptides having more than 30 amino acid residues.

11. Use of proline specific endoprotease of claim 4-6, 9 or 10 whereby the proline specific endoprotease is an *Aspergillus*, preferably an *Aspergillus niger* enzyme.

USE OF PROLINE SPECIFIC ENDOPROTEASES TO HYDROLYSATE PEPTIDES AND PROTEINS

ABSTRACT

The present invention describes a process for the proteolytic hydrolysis of a peptide or protein, said peptide comprising 4 to 40, preferably 5 to 35, aminoacids and whereby the amino acids of the peptide or protein are for at least 30%, preferably for at least 40%, proline whereby said peptide or protein is hydrolysed by a proline specific endo protease at a pH of 6.5 or lower to hydrolyse of said peptide or protein.

Figure 1

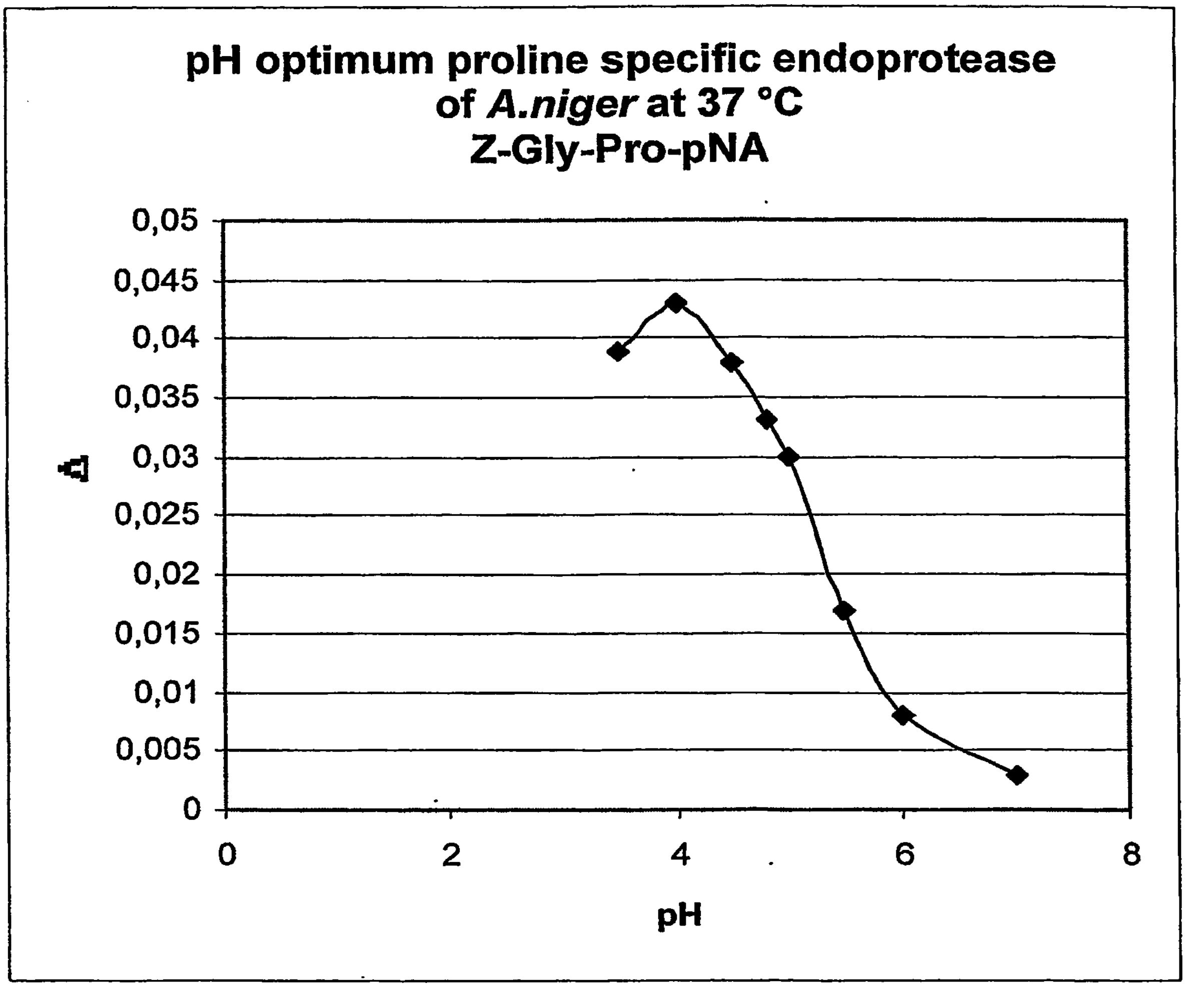
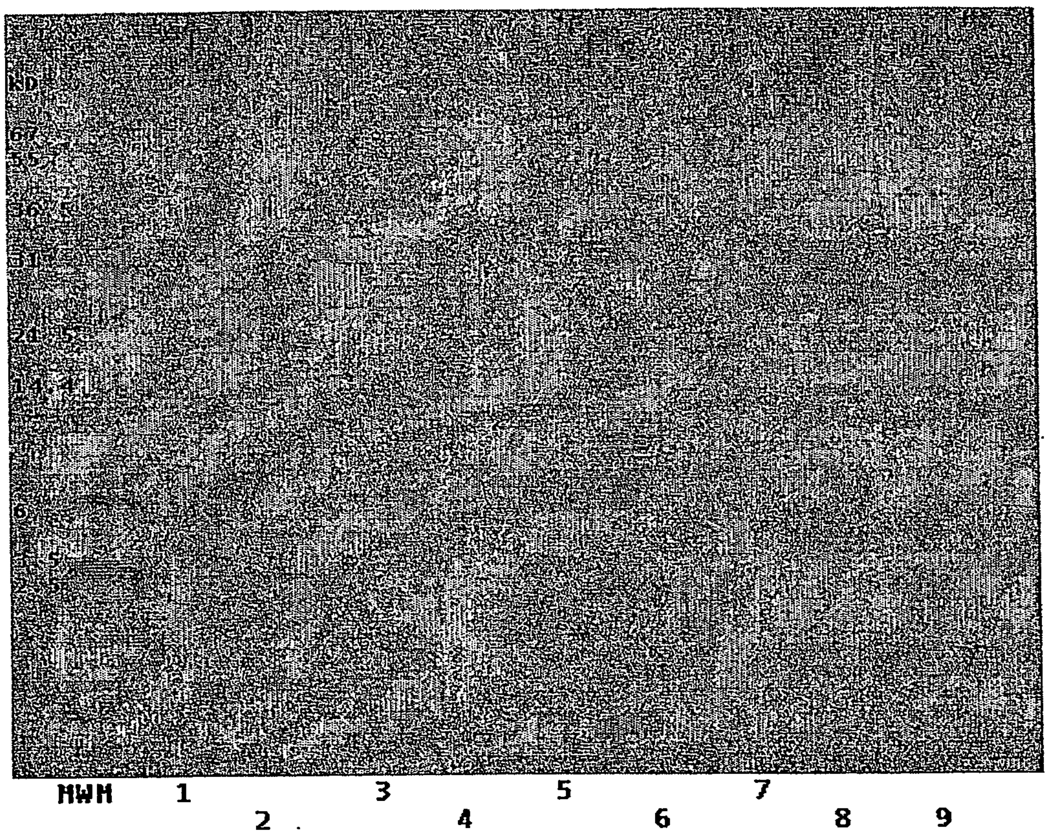

pH optimum proline specific endoprotease
of A.niger at 37 °C
Z-Gly-Pro-pNA
0,05
0,045
0.04
0.035
0.03
0,025
0.02
0,015
0,01
0,005
0
0
2
4
6
8
pH

Figure 2



MWM = molecular weight markers

Lanes 1, 3, 5, 7: ovalbumine after 0, 4.75, 20,5 and 27.75 hours of incubation respectively (5µl loaded); lanes 2, 4, 6, 8: 27-mer peptide after 0, 4.75, 20,5 and 27.75 hours of incubation respectively (5µl loaded). Lane 9: 27-mer at 0 hours (2µl loaded).